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Humoral-Phagocyte Axis of Immune Defense in Burn Patients

Chemoluminigenic Probing

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• Both serum opsonic capacity and granulocyte oxygenation activity were measured in 35 burn patients during their course of therapy. The microbicidal action of granulocytes is effected via the metabolic generation of oxygenating agents; introduction of chemoluminigenic substrates, such as luminol or dimethyl biacridinium dinitrate, allows ultrasensitive measurement of phagocyte oxygenation activity. Serum opsonic capacity can also be assayed by measuring the rate of activation of phagocyte oxygenation activity. Alterations in granulocyte oxygenation activity were observed in individual patients in temporal association with changes in clinical condition, and sepsis was associated with a marked decrease in activity. An initial depression in opsonic capacity was noted at the time of admission of patients with major burns, more than 40% total body surface. Thereafter, depression of opsonic capacity was temporally associated with sepsis in individual patients. Chemoluminigenic probing provides a rapid, sensitive, and objective method for assessing the status of the humoral-phagocyte axis, and as a clinical laboratory technique is particularly applicable for monitoring patient populations in which sepsis is prevalent.

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Despite our expanded arsenal of antibiotics, septic complications continue to be a major contributor to morbidity and mortality in the burn patient. Such infections, commonly caused by opportunistic pathogens, imply alteration of host resistance to infection. The primary host defense against most bacterial and some fungal infections is provided by an information-effector system termed the humoral-phagocyte axis of immunity. The humoral element, composed of opsonic proteins, serves as the information mechanism that triggers a sequence of immunochemical events leading to phagocyte microbicidal activity.¹⁻³ Alterations of both elements of the humoral-phagocyte axis have been described in the burn patient.⁴⁻¹¹

Currently available techniques for testing humoral and phagocyte function are time consuming, expensive, and require a high degree of technical expertise. Chemoluminigenic probes (CLP), such as luminol and dimethyl biacridinium dinitrate (DBA), have made possible the development of an ultrasensitive, rapid, inexpensive, and

nondestructive method for quantification of phagocyte microbicidal metabolism based on measurement of the photon emission associated with the oxygenation of these high-quantum-yield substrates.^{3,12,13} The results and interpretation of our initial research using CLP techniques for assessment of the humoral-phagocyte axis of immunity in burn patients are described in this report.

PATIENTS AND METHODS

Patient Data

The present study included eight healthy control subjects and 35 burn patients. All of the patients sustained their burn injuries in the same accident and all were admitted to the Institute of Surgical Research, Fort Sam Houston, Tex, on the third postburn day (PBD). The patients were all previously healthy men ranging in age from 20 to 22 years. Nine patients had 2% to 18% total body surface (TBS) burns; eight patients had 21% to 36% TBS burns; ten patients had 42% to 56% TBS burns; and eight patients had 62% to 93% TBS burns. The control subjects included five men and three women ranging in age from 21 to 34 years. Of the 35 patients studied, seven died. The charts of three of these nonsurviving patients were reviewed, and the significant clinical changes and therapeutic interventions are presented chronologically for correlation with laboratory measurements of granulocyte oxygenation activity and serum opsonic capacity. All blood specimens were obtained between 6 and 7 AM. Total and differential leukocyte counts were performed on the blood specimens to calculate the specific granulocyte activity for each patient. Informed consent was obtained from patients and control subjects, and the studies were conducted in accordance with Army Medical Research and Development Command project number 3A161101A91C.

Granulocyte Oxygenation Measurement

Whole blood obtained by venipuncture was collected in tubes with edetic acid for standard blood counts. A 0.1-mL aliquot was taken from the well-mixed specimen and added to a tube containing 0.9 mL of phosphate-buffered saline solution, pH 7.2, to yield a one to ten (1:10) dilution; 100 μ L of the 1:10 diluted whole blood were then added to sterile, siliconized glass counting vials (24 mL capacity) containing 1.68 mL of barbital- (Veronal) buffered saline solution with calcium and magnesium ions plus albumin (0.1% w/v) and glucose (0.1% w/v, pH of 7.2).¹⁴

Two CLPs were employed: luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione), of which a 1mM stock solution was prepared in dimethyl sulfoxide (DMSO); and dimethyl biacridinium dinitrate (DBA; Lucigenin, 10,10'-dimethyl-9,9'-biacridinium dinitrate), of which a 1mM stock solution was prepared in water. Just prior to use, the CLP stock solutions were diluted with water to yield 5 μ M substocks, and 0.2 mL of either substock was added to each vial. The final CLP concentration was 0.5 μ M, and the final pH of the suspension was 7.2.

The vials were then placed in the counter and luminescence

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intensity measurements were taken every 13 minutes. After three measurements of prestimulation background luminescence, 20 μ L of either chemical or particulate stimulant were added per vial within 30 s of the time-zero count. A 5mM stock solution of phorbol myristate acetate (PMA; phorbol 12-myristate-13-acetate) was prepared in DMSO. This stock was diluted with water to yield a 2.5 μ M substock solution. Addition of 20 μ L of substock to the vial yielded a final concentration of 25nM PMA. Zymosan A was suspended in normal saline solution (250 mg/dL), heated to 90 °C for 30 minutes, and the cooled suspension was opsonified with an equivalent volume of guinea pig complement (250 total hemolytic complement units/mL). After 30 minutes of incubation, the suspension was centrifuged, the serum decanted, and normal saline solution added to yield opsonified zymosan, 50 μ g/20 μ L.

Dimethyl sulfoxide does not influence granulocyte function at the concentration employed in these studies. Light absorption by hemoglobin presents a problem with regard to direct measurement of chemoluminescence (CL) from phagocytes using whole blood specimens. This problem was sufficiently overcome by diluting the whole blood specimen 1:200 and adjusting the concentration of the CLP to provide the necessary sensitivity for oxygenation measurement.

Serum Opsonic Capacity Measurement

Blood was obtained from healthy volunteers, and the leukocyte-rich plasma was isolated following heparin-dextran sedimentation. After hypotonic lysis of the remaining erythrocytes (0.2% saline for 15 s) and two additional washes in phosphate-buffered saline, total and differential counts were taken. The volume was adjusted to yield 1,000 polymorphonuclear leukocytes per microliter, and 25 μ L of this polymorphonuclear leukocyte suspension was added to vials containing 1.75 mL of complete barbital buffer plus 0.2 mL of 5 μ L luminol as described in the previous section. The patient or control serum to be tested was diluted, and a different dilution was added to each vial to allow titration of activity. The vials were then placed in the counter and CL intensity measurements were taken every seven minutes. After three prestimulation measurements, 20 μ L (50 μ g) of zymosan suspension (unopsonified) were added per vial within 30 s of the time-zero count.

Single Photon Counting

Chemoluminescence was quantified at room temperature (22 °C) using the single photon counting capacity of a scintillation counter equipped with bialkali photomultiplier tubes and operated in the out-of-coincidence mode using the tritium channel settings.¹⁴ The raw CL intensity values were converted to photons per minute by multiplying the relative counts per minute by a photon conversion factor, 14. This factor was established by calibrating the counter with a known blue photon emitter as described by Seliger.¹⁵ Values for integral CL response were calculated from the CL intensity data by trapezoidal approximation.

RESULTS

Granulocyte Oxygenation Activity

The activation of polymorphonuclear leukocyte metabolism associated with phagocytosis or chemical stimulation results in the generation of oxygenating agents capable of exerting microbicidal action. Introduction of bystander substrate molecules, whose oxygenation results in a high yield of electronically excited products, allows ultrasensitive measurement of phagocyte oxygenation activity through quantification of the luminescence resulting from relaxation of these excited products. Appreciation of the

utility of CLP can be gained by perusal of Fig 1. In Fig 1, left, CL intensity is plotted against time. The portion of the curves to the left of time zero depicts prestimulation activity. The kinetics and magnitude of the CL responses from this patient's granulocytes show a pattern consistent with normal. Note that the pattern of oxygenation activity depends on the stimulus and CLP employed. Characteristically, the granulocyte response to opsonified zymosan is relatively rapid as measured by luminol oxygenation, whereas DBA oxygenation in response to stimulation by PMA is slower but sustained. In Fig 1, right, the data are depicted as the integral or cumulative CL plotted against time. The integral values were calculated from the intensity data by trapezoidal approximation.

The Table was constructed from CL data generated by measurements as described in Fig 1. The integral CL for the two-hour poststimulation time interval was found to be a linear function of the number of granulocytes present in the whole blood specimen tested. The data of a representative experiment are depicted in Fig 2. Using opsonified zymosan as the stimulant and luminol as the CLP, the total number of granulocytes in the 10- μ L blood specimen is plotted against the integral photons measured for the two-hour poststimulation interval. As reported in the Table, for most of the experiments where opsonified zymosan was the stimulus and luminol was the CLP, the coefficient of determination (r^2) was approximately .8, indicating a high degree of correlation. The skewing effect of a few individuals with abnormal granulocyte function was responsible for poor correlation ($r^2 < .8$) in some experiments. For example, on PBD 13, if only the surviving patients ($n = 22$) were considered in the calculation, the function becomes as follows: integral photons $\times 10^3/2$ hr = $2.5 \times (\text{number of granulocytes}) - 2.9$, with an r^2 of .87.

Integral CL also correlated with the number of granulocytes present in the whole blood specimen when PMA was the stimulant and DBA was the CLP. However, the coefficients of determination were not as high as those obtained using opsonified zymosan plus luminol. The lower r^2 values reflect the skewing effect of individual patients or groups of patients with low specific activity. This effect is graphically depicted in the plot of DBA-dependent integral CL against the number of granulocytes stimulated with PMA presented in Fig 3. The r^2 for the linear regression, plotted as the unbroken line, is .53 when all patients and controls are considered in the calculation. However, when those patients with greater than 60% TBS burn are excluded from consideration, the equation relating CL to granulocyte count becomes as follows: integral photons $\times 10^3/2$ hr = $1.0 \times (\text{number of granulocytes}) \times 1.8$, with an n of 18 and an r^2 of .87. This new plot is indicated by the broken line in Fig 3.

Granulocyte oxygenation activity is reported as granulocyte oxygenation index (GOI). The GOI is calculated by dividing the actual measured granulocyte oxygenation activity of a given specimen by the calculated activity based on values obtained for control subjects and patients with less than 40% TBS burn. For example, patient 20 on

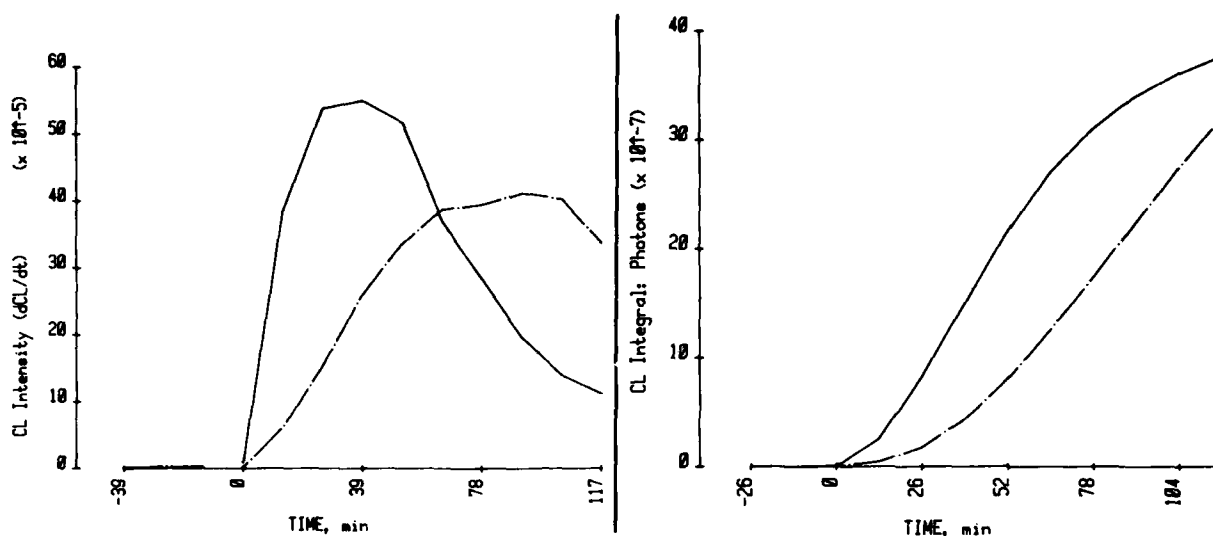


Fig 1.—Plots of intensity (left) (measured in photons per minute) and integral (right) chemoluminescence (CL) data against time, for patient 19 on postburn day 13; burns were 42% of total body surface. Whole blood used was 10 μ L in 2 mL complete barbital buffer; chemoluminogenic probe was luminol, 0.5 μ M (solid line), or dimethyl biacridinium dinitrate, 0.5 μ M (broken line). Patient's whole blood specimen contained 21,800 leukocytes per microliter with 58% segmented and 7% band neutrophils, 3% eosinophils, 4% monocytes, and 28% lymphocytes. Twenty microliters of either 25nM phorbol myristate acetate (broken line) or serum opsonified zymosan (solid line) 2.5 μ g/ μ L were added at time zero. Cumulative integral CL was calculated from intensity data by trapezoidal approximation.

| Specific Oxygenation Activity of Granulocytes in Whole Blood* | | | | | | | | | |
|---|--------------|-----------------------------|-----|------|----------------|--------------------------|-----|------|----------------|
| | Postburn Day | Stimulant: OZ; CLP: Luminol | | | | Stimulant: PMA; CLP: DBA | | | |
| | | n | m | b | r ² | n | m | b | r ² |
| Control plus 2% to 36% TBS burn | 4 | 10 | 3.3 | -5.6 | 0.84 | 10 | 1.8 | 0.6 | 0.54 |
| | 11 | 16 | 3.6 | -5.9 | 0.85 | 16 | 1.1 | 2.5 | 0.83 |
| | 13 | 12 | 3.4 | -7.2 | 0.96 | 12 | 1.0 | | 0.45 |
| | 18 | 12 | 2.8 | -5.9 | 0.82 | ND | ND | | ND |
| | 20 | 13 | 2.6 | -4.4 | 0.78 | 13 | 0.6 | | 0.54 |
| | 25 | 13 | 2.3 | -2.6 | 0.86 | 13 | 0.9 | | 0.83 |
| | 27 | 9 | 2.0 | -0.9 | 0.88 | 9 | 0.9 | 2.3 | 0.84 |
| 42% to 93% TBS burn | 4 | 12 | 2.3 | -2.3 | 0.87 | 12 | 1.1 | -0.6 | 0.60 |
| | 11 | 12 | 3.1 | -6.2 | 0.78 | 12 | 1.0 | -1.2 | 0.89 |
| | 13 | 16 | 1.1 | 12.2 | 0.65 | 16 | 0.7 | 2.9 | 0.70 |
| | 18 | 13 | 1.8 | 1.0 | 0.65 | ND | ND | ND | ND |
| | 20 | 13 | 2.0 | -2.9 | 0.81 | 13 | 0.5 | 1.3 | 0.48 |
| | 25 | 12 | 3.2 | -8.1 | 0.81 | 12 | 1.5 | -4.3 | 0.66 |
| | 27 | 12 | 2.5 | -1.7 | 0.75 | 12 | 0.7 | 3.5 | 0.41 |
| Control plus all patients | 4 | 22 | 2.3 | -2.4 | 0.88 | 22 | 1.0 | 2.0 | 0.51 |
| | 11 | 28 | 3.1 | -4.0 | 0.84 | 28 | 0.8 | 2.9 | 0.80 |
| | 13 | 28 | 1.4 | 6.5 | 0.67 | 28 | 0.7 | 4.9 | 0.59 |
| | 18 | 25 | 2.0 | -0.6 | 0.78 | ND | ND | ND | ND |
| | 20 | 26 | 2.1 | -2.2 | 0.82 | 26 | 0.5 | 2.0 | 0.49 |
| | 25 | 25 | 3.1 | -6.8 | 0.83 | 25 | 1.4 | -1.5 | 0.65 |
| | 27 | 21 | 2.5 | -2.5 | 0.79 | 21 | 0.7 | 3.5 | 0.53 |

* Defined as integral photons $\times 10^7 / 2 \text{ hr} = m \times (\text{number of granulocytes}) + b$. OZ indicates opsonified zymosan, 50 μ g/2 mL; CLP, chemoluminogenic probe; PMA, phorbol myristate acetate, 25nM; luminol, 0.5 μ M; DBA, dimethyl biacridinium dinitrate, 0.5 μ M; n, number of specimens; m, slope of linear function; b, y intercept of linear function; TBS, total body surface; ND, not done. These data were obtained using the conditions of testing described in Fig 1.

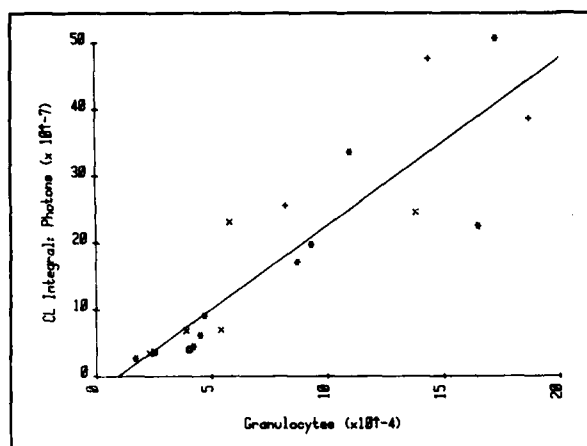


Fig 2.—Integral chemoluminescence (CL) response for 117-minute interval following stimulation plotted against total number of granulocytes in 10- μ L specimen of whole blood tested in 2 mL complete barbitol buffer; postburn day 27. Granulocyte count included segmented, band, and metamyelocytic neutrophils and also eosinophils. Quantification of integral CL was as described in Fig 1. Patient specimens were divided into four groups: (1) controls plus patients with 3% to 18% total body surface (TBS) burns (\emptyset); (2) patients with 21% to 36% TBS burns (x); (3) patients with 42% to 56% TBS burns (*); and (4) patients with 61% to 93% TBS burns (+). Chemoluminescent probe was luminol, 0.5 μ M; stimulant, opsonified zymosan, 50 μ g.

PBD 20 had a granulocyte count of $1.3 \times 10^5/10 \mu\text{L}$ whole blood yielding 1×10^8 photons during the two-hour interval following stimulation by opsonified zymosan with luminol as CLP. Using the equation established for "control plus 2% to 35% TBS burn" patients on PBD 20 as presented in the Table, the granulocytes of patient 20 are calculated to yield 3×10^8 photons during this poststimulation interval. Therefore, his GOI is $\frac{1}{3}$ or 0.33.

Serum Opsonic Capacity

The humoral or information aspect of acute immune defense can also be tested by a modification of the CLP approach. When serum concentration is the only variable, opsonic capacity can be expressed as the rate of activation of phagocyte oxygenation activity and can be measured by CLP. Serum opsonification of zymosan is a measure of nonspecific opsonic capacity; that is, it is considered to proceed via the alternative pathway of complement activation. The titrations of zymosan (nonspecific) serum opsonic capacity for a control subject and a patient are depicted in Fig 4 and 5, respectively. In the figures, the integral CL responses are plotted against time. Note that the quantity and source of the serum are the only variables.

The relationship between quantity of serum and opsonic capacity is not linear and more closely approximates the sigmoidal relationship previously established for complement hemolytic assays.¹⁴ This sigmoidal relationship is illustrated by plotting the integral CL data of Fig 4 and 5 against the quantity of serum tested as done in Fig 6. In Fig 6, the ordinate is presented as the log of the fraction:

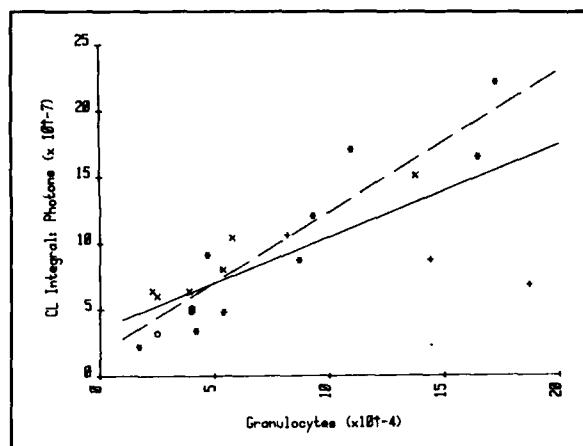


Fig 3.—Plot of integral chemoluminescence (CL) for 117-minute interval following stimulation with 25nM phorbol myristate acetate (PMA). Conditions are same as those described for Fig 2 except that PMA was stimulant and dimethyl biacridinium dinitrate, 0.5 μ M, was chemoluminescent probe. Solid regression line is obtained when all patients are considered; broken regression line is obtained when 61% to 93% total body surface burn patients are excluded from calculation.

integral CL for the specimen divided by the difference, maximum integral CL for all specimens minus integral CL for the specimen; thus, at 50% stimulation of granulocyte oxygenation activity, the ordinate value will be zero. Therefore, under the stated conditions of testing, the quantity of serum required for 50% activation can be defined as a nonspecific or zymosan opsonic 50 (Op 50) unit. For the control serum, 7.8 μL were equivalent to 1 Op 50 unit; for the patient, 22.6 μL of serum were required for 1 Op 50 unit. Thus, the control subject contained 128 Op 50 units/mL serum, and the patient contained 44 Op 50 units/mL serum. The mean \pm SD for seven control sera was 123 ± 19 Op units/mL.

Temporal Studies of Individual Patients

The clinical changes that occurred in three nonsurviving patients, as well as changes in total leukocyte and granulocyte count, and the changes across time of serum opsonic capacity and GOI, as measured by the CLP techniques described, are depicted in Fig 7, 8, and 9.

COMMENT

The primary phagocyte of the humoral-phagocyte axis of acute host defense against infection is the granulocyte, and the metabolically dependent generation of oxygenating agents is essential for effective granulocyte microbicidal action.^{1,3} The CLP techniques employed in the present study were designed to measure the oxygenation activity of stimulated phagocytes. Stimulation was effected using either serum-opsonified zymosan, a phagocytosable particulate stimulus, or PMA, a chemical stimulus. Oxygenation activity was further differentiated by use of different CLP. The luminol CL reaction is a dioxygenation and in the granulocyte reflects myeloperoxidase-associated activity.

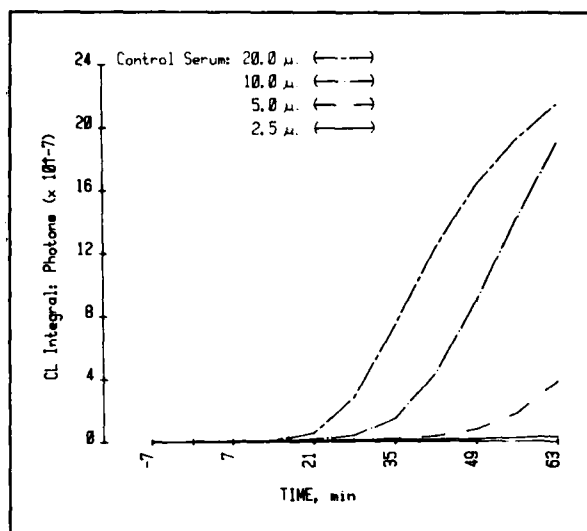


Fig 4.—Plot of cumulative integral chemoluminescence (CL) against time from isolated polymorphonuclear leukocytes, 25,000 in 2 mL complete barbital buffer, with luminol, 0.5 μ M, as chemoluminescent probe. In experiment, only variable is quantity or dilution of normal, control serum present per vial. Opsonification was initiated at time zero by addition of 20 μ L (50 μ g) unopsonified zymosan.

The DBA reaction is a reductive dioxygenation and in the granulocyte can provide a measure of superoxide generation.¹³

Specific oxygenation activity was normal in many patients where bands and metamyelocytes accounted for greater than 70% of the total granulocyte count; therefore, segmented, band, and metamyelocytic neutrophils plus eosinophils were included in the calculation of specific oxygenation activity. Grogan has also reported that the juvenile neutrophils in burn patients had normal bactericidal capacity.¹⁶

The tabulated results of specific oxygenation measurements of granulocytes from the burn patients are presented in the Table. Specific activity was generally lower in granulocytes from the greater than 40% TBS burn group as compared with control subjects plus patients with less than 40% TBS burns. However, many patients with major burn injuries had specific oxygenation activities comparable with or greater than control values. Specific activity varied with the clinical state of the patient. Figures 7, 8, and 9 depict the temporal relationships of oxygenation activity, presented as the GOI, to clinical condition in three nonsurviving patients. In most instances, the GOI paralleled opsonic capacity. Measurements of GOI in patient 20 were approximately 1.0 on PBD 4 and 6 and decreased to 0.8 in temporal relation to a decrease in opsonic capacity on PBD 11. The GOI rose to greater than 1.0 on PBD 13 and 18, but on PBD 20 a profound decrease to below 0.4 was associated with a precipitous decrease in opsonic capacity and verified *Proteus* septicemia. This patient died two days later.

A somewhat different pattern was observed in patient 28. The GOIs were consistently low for the PBD 4 and 6

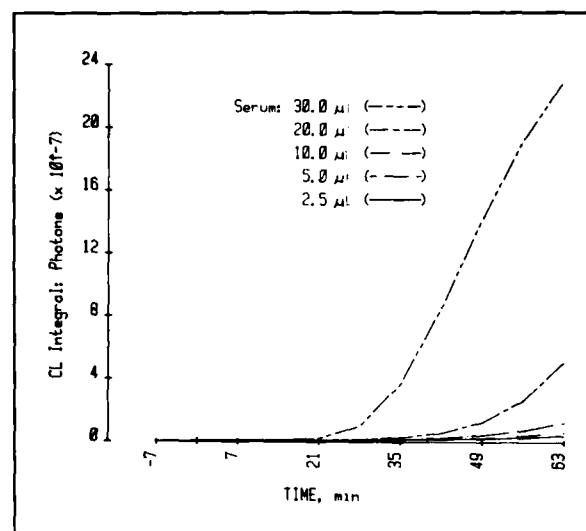


Fig 5.—This plot is same as described in Fig 4 except that serum of patient 13 is titrated (postburn day 5; 56% total body surface burn).

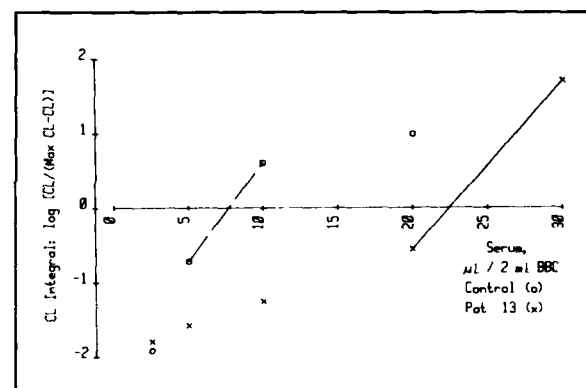


Fig 6.—Integral chemoluminescence (CL) response for 63-minute poststimulation interval expressed as log (individual specimen CL divided by difference of maximum CL for all specimens minus individual specimen CL) plotted against quantity of serum present per vial. This figure is constructed from data presented in Fig 4 and 5. When y value is zero, x value is that quantity of serum required for 50% maximum integral CL from granulocyte suspension. BBC indicates complete barbital buffer; Pat, patient.

measurements in temporal association with persistently low opsonic capacity. On PBD 13, the granulocyte count rose to 54,500/ μ L following the occurrence, and steroid treatment, of an episode of clinical sepsis as indexed by otherwise unexplained hypotension and hyperthermia. The GOI on PBD 13 was approximately 0.4, and the patient died on PBD 16. A GOI below 0.4 was also measured in patient 13 on PBD 25 in temporal association with burn-wound infection and profound depression of opsonic capacity.

These findings suggest a relationship between granulocyte oxygenation activity and state of infection. However, in the present study, frequency of sampling was limited to two specimens per week, and it is not possible to determine

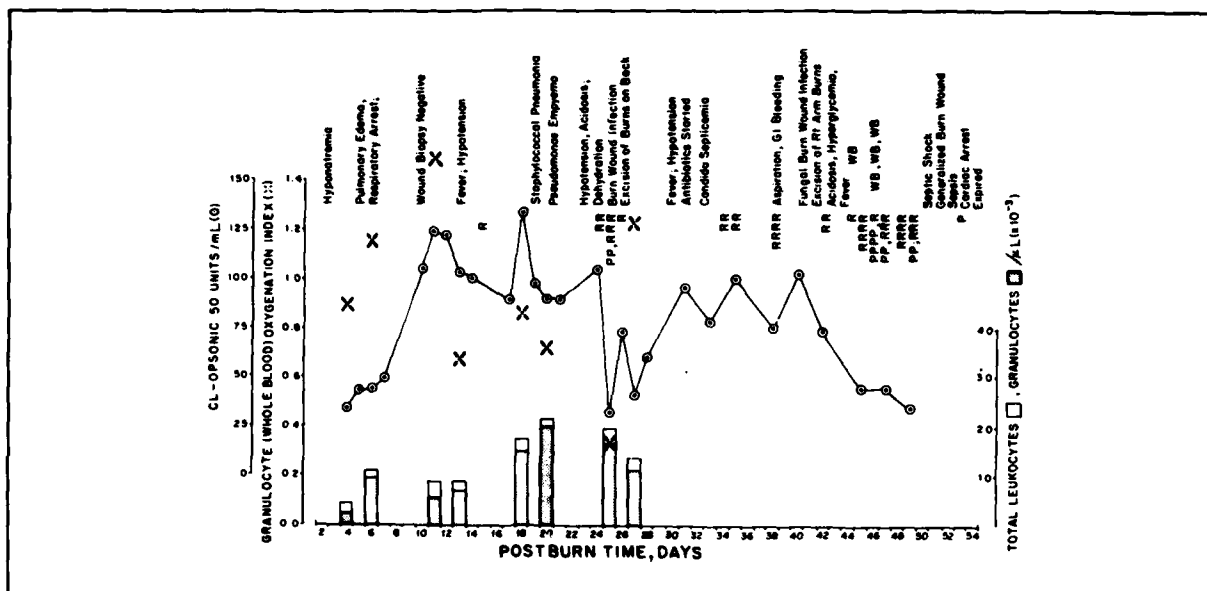


Fig 7.—Temporal plot of laboratory and clinical data for patient 13, with 56% total body surface burns. Significant changes in clinical course are presented at top of figure. Transfused blood elements are represented as: WB, 500 mL whole blood; R, 250 mL packed erythrocytes; and P, 210 mL fresh frozen plasma. Left-hand ordinates present value of serum opsonic-50 capacity, and also value of granulocyte oxygenation index on day tested. Right-hand ordinate refers to bar graphic and numerically presents leukocyte and granulocyte counts. GI indicates gastrointestinal; CL, chemoluminescence.

Fig 8.—Temporal plot of laboratory and clinical data for patient 20 presented as described in Fig 7: 85% total body surface burns.

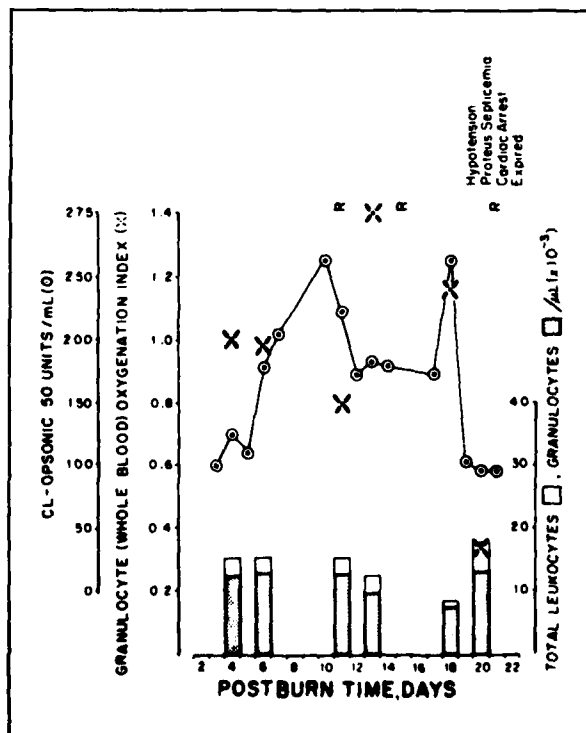
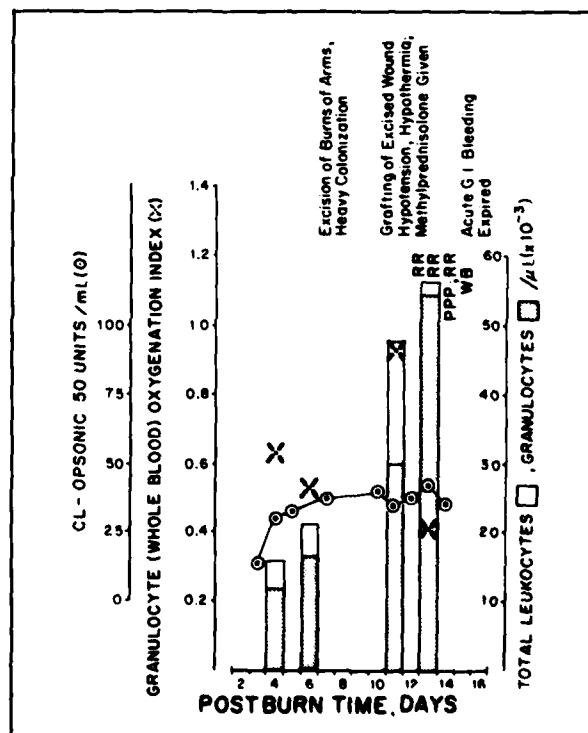


Fig 9.—Temporal plot of laboratory and clinical data for patient 28 presented as described in Fig 7; 64% total body surface burns.



whether the relationship observed is one of cause or effect. Possible causes of decreased GOI are numerous and include hormonal fluctuations, such as increased circulating levels of catecholamines and corticosteroids and decreased levels of thyroid hormone; fungal or bacterial toxemia; serum inhibitors; and circulating antigen-antibody complexes. At present there is no convincing evidence to incriminate any single mechanism.¹⁷

The serum opsonic capacities for these nonsurviving patients were also measured throughout the course of hospitalization, and the results are plotted as zymosan opsonic 50 units per milliliter of serum. As depicted in Fig 7, the opsonic capacity of patient 13 was markedly depressed during the first postburn week with values ranging from 35 to 50 Op 50 units/mL serum, but by PBD 11, opsonic capacity was within the range of normal controls. After PBD 12, opsonic capacity followed a variable course in association with multiple clinical episodes of infection. A profound decrease in activity to 33 Op 50 units/mL was measured on PBD 25 in association with documented burn wound infection. During the period from PBD 28 to 40, opsonic capacity was measured intermittently, with fluctuations in activity observed in association with *Candida* septicemia and aspiration. Following PBD 40, a progressive decrease in opsonic capacity was measured in association with fungal burn wound infection. During the following nine days, an insidious decrease in opsonic capacity paralleled preterminal clinical deterioration and was not reversed by administration of multiple units of whole blood, packed erythrocytes, and fresh frozen plasma.

As shown in Fig 8, patient 20 had 101 Op 50 units/mL serum on PBD 4, a value only slightly below the mean \pm SD (123 ± 19) of the control sera tested. By PBD 7, the serum capacity had risen to 204 Op 50 units/mL, and by

PBD 10 it reached a maximum of 263 Op 50 units/mL. Even though at levels near control levels, the serum opsonic capacity measurements on PBD 3, 4, and 5 were low relative to the patient's maximum capacity, and on PBD 19 and 20 opsonic capacity fell to a level of 97 Op 50 units/mL in association with hypotension and *Proteus* septicemia. Patient 28 had exceptionally low serum opsonic capacity throughout his relatively short clinical course as depicted in Fig 9. Only 15 Op 50 units/mL serum were measured on PBD 3, and opsonic capacity remained below 43 Op 50 units/mL until he died on PBD 16.

Relative or absolute depression of opsonic capacity was noted on admission in all three patients. Secondary depression in serum opsonic capacity was temporally associated with sepsis, and control of sepsis was associated with improvement in capacity. Initial depression may reflect the activation and consumption of complement by heat altered tissue¹⁸ or it may reflect transeschar exudation of complement.¹⁹ Secondary depression in activity may be related to the presence of a circulating inhibitor²⁰ or may be related to complement consumption secondary to sepsis.⁴

The CLP techniques employed in this study are currently undergoing further improvement and testing for eventual use as a routine clinical laboratory technique. Chemoluminescent probing promises to provide a rapid, sensitive, inexpensive, and objective method for assessment of both aspects of humoral-phagocyte immunity. Such information is important as timely detection of a decrease in either humoral or phagocytic function would alert the clinician to probable sepsis and thus prompt a thorough search for the causative infection and early treatment.

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Discussion

J. WESLEY ALEXANDER, MD, Cincinnati: We have looked at the problem of opsonization and neutrophil function for several years now and have made thousands of observations in hundreds of patients. We have shown that there is a correlation between the ability of neutrophils to ingest and kill *Staphylococcus aureus* bacteria, which is killed through oxidative pathways in neutrophils, and the incidence of bacteremia in a variety of patients, primarily burn patients.

There is also an important correlation with the ability of the patient's serum to opsonize bacteria, although this is less prominent than the ability of neutrophils to kill. In situations where there is normal neutrophil function, abnormal opsonization, at least as seen clinically, does not seem to predispose to bacterial infection to a significant degree.

We have also shown that the incidence of bacteremia does not correlate with the ability of the neutrophils to kill *Escherichia coli*, for reasons that are not known to us, nor does there seem to be a significant correlation with chemotaxis. Thus, different functions of the neutrophil certainly may have different biologic importance.

Though these studies have allowed us to examine groups of patients and to determine various treatments and their effect on the host defense mechanisms, they have not been clinically useful for individual patients. They do not help us to determine when a patient may be particularly susceptible to infection in time to alter therapy because it takes so long to get the answers.

The tests that Drs Allen and Pruitt have described are extreme improvements on other CL tests, which should allow us to apply prospective measurements to alter therapy. This study, though exciting, is preliminary, and there needs to be much more work particularly relating to the need for control of neutrophils and the influence of the erythrocyte-neutrophil ratio. Another question that might make the test even more useful in looking at an individual patient is whether it can measure both opsonization and neutrophil function in the same blood sample. There might be a limitation on that because of the necessity of diluting out the erythrocytes.

DONALD TRUNKEY, MD, San Francisco: What evidence do the authors have that whole blood is an adequate test for neutrophil function? Are they also measuring other monocyte phagocytosis?

I was intrigued by their patient data and particularly the biphasic course of the deterioration of neutrophil function, which parallels very closely what we have shown with regard to the relative increase of T suppressor cells. Did they measure macrophage function in these patients? We think the relative increase in T suppressor cells may be related to an inhibitory macrophage, and we think that they may also control neutrophil function.

THOMAS K. HUNT, MD, San Francisco: We know that there is a biologic process behind CL that is P_{O_2} dependent, that is, there can be none in the absence of oxygen. I assume first that they did their studies in media equilibrated with air. White blood cells that are actually killing bacteria are operating in a much lower P_{O_2} . Our data would suggest that it is somewhere between 0 and 30 mm Hg. I wonder if they have had the opportunity to plot the curve of the P_{O_2} dependence of CL activity and if it might not be possible that their separations might be clearer if they chose a more physiologic P_{O_2} in which to test these cells.

M. LANSER, MD, Albany, NY: In our laboratory, we have been looking at opsonic fibronectin in the postburn period and its relationship to postburn sepsis. We have found that the changes in opsonic fibronectin concentrations following burn very much parallel the changes in the CL response as the authors described in their postburn patients. Zymosan, as well as other particulate activators of the alternative complement pathway, may require fibronectin for their ingestion by phagocytes. The opsonic defect for *S aureus* in postburn serum mentioned by Dr Alexander may likewise result from opsonic fibronectin deficiency. We have recently found that fibronectin-free rat or human serum is markedly deficient in its ability to support phagocytosis of *S aureus* by both rat and human neutrophils in vitro. Thus, though they have equated the opsonic activity as measured by CL with alternative complement activity, perhaps this may be related to opsonic fibronectin levels. Have they, therefore, measured alternative complement activity more directly in their plasma samples?

DR ALLEN: In response to Dr Alexander's questions, regarding the erythrocyte-leukocyte ratio, in the experiments presented, the whole blood was diluted by 100 to 200 to minimize the light-quenching effect of erythrocytes, and the CLP concentration was adjusted to provide optimum detection of phagocyte oxygenation activity at that dilution. Under the conditions, hemoglobin quenching of luminescence was still detectable, but the effect was small, and therefore, the hematocrit values were not considered in the calculation.

Regarding simultaneous measurements, at present we are investigating the simultaneous measurement of opsonic and phagocyte function as a screening procedure. If an abnormal activity is detected, opsonic activity and phagocyte function could be separately measured in an additional run.

In response to Dr Trunkey's questions, regarding macrophage function, monocytes are present in the whole blood preparations, and these phagocytes do yield CL. However, the monocyte luminescence response to stimulation with opsonified zymosan is less than one half that obtained from granulocytes, and when monocytes and granulocytes are considered together in the calculations, the plots of the data are essentially the same. With regard to studies of macrophage function, I assume he means in vitro conversion of monocytes to macrophages. We have not investigated this area, but it might be interesting to do so.

In answer to Dr Hunt's question, regarding P_{O_2} dependence, oxygen is required for phagocyte luminescence. Our unpublished investigations indicate that the K_m (Michaelis constant) of the phagocyte oxidases for oxygen is very small; that is, oxygen utilization can proceed at very low P_{O_2} . In the present study, the effect of oxygen as a rate-limiting factor was minimized by testing a small number of phagocytes and increasing the surface-volume ratio of the suspending medium to promote oxygen solubilization. However, the measurement of phagocyte P_{O_2} requirement would also provide a valid method for assessing function.

In response to Dr Lanser's question, regarding fibronectin, we have not investigated the role of fibronectin in opsonification, but such a study should be conducted. Support for the role of complement in zymosan opsonification is based on heat-lability and cation-chelation studies.

